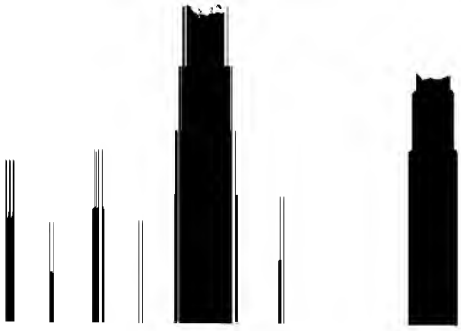
ANTIBODY TECHNIQUES

EDITED BY

Vedpal S. Malik
U.S. Department of Agriculture, APHIS
Hyattsville, Maryland

Erik P. Lillehoj
Department of Biochemistry and Molecular Biology
The George Washington University
School of Medicine and Health Sciences
Washington, D.C.

Academic Press
San Diego New York Boston
London Sydney Tokyo Toronto



Academic Press, Inc.
A Division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495

United Kingdom Edition published by
Academic Press Limited
24-28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

Antibody techniques / edited by Vedpal S. Malik, Erik P. Lillehoj.
p. cm.
Includes bibliographical references and index.
ISBN 0-12-466460-1
1. Immunoassay--Laboratory manuals. 2. Immunoglobulins--Laboratory manuals. 3. Monoclonal antibodies--Laboratory manuals. 4. Immunochemistry--Laboratory manuals. I. Malik, Vedpal S. II. Lillehoj, Erik P.
QP 519.9.I42A56 1994
574.2'93--dc20 94-16438
CIP

PRINTED IN THE UNITED STATES OF AMERICA
94 95 96 97 98 99 EB 9 8 7 6 5 4 3 2 1

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Flow Cytometry and Fluorescence-Activated Cell Sorting

Hyun S. Lillehoj and Alison Martin

I. INTRODUCTION

Flow cytometry (FCM) is a method that measures the chemical and/or physical characteristics of particles as they pass through a detection device in a fluid stream. In immunofluorescence FCM, properties of the particles, usually cells, are assessed by an antibody molecule conjugated to a fluorochromatic dye. Fluorescence-activated cell sorting (FACS) is a process in which viable cells with pre-defined properties are separated by FCM. Biological particles that have been analyzed by FCM and FACS include prokaryotic and eukaryotic cells, normal and tumor cells, viruses, fungi, parasites, cellular organelles, and chromosomes. Commercially available flow cytometers include sophisticated optical, electronic, and nuclear radiation sensors as well as computers for data analysis.

With the advent of monoclonal antibodies against cell-surface antigens, FCM has evolved as a standard research tool. FCM analysis is useful not only to quantify distinct subpopulations of cells, but also to characterize the host's antibody response to bacteria, virus, or parasites. Most current immunological applications of flow cytometry relate to identification and quantification of heterogeneous cell populations. Also, analyses of physical characteristics (cell size and shape, cytoplasmic granularity, protein fluorescence, DNA content) as well as functional characteristics (enzyme activity, redox state, intracellular

Antibody Techniques

receptors, endocytosis, surface receptors, membrane-bound and cytoplasmic Ca^{2+}, intracellular pH) are possible using the flow cytometer.

One of the most popular applications of FCM is in the biological sciences, especially immunology, for isolation and characterization of subpopulations of cells within heterogeneous populations. Most successful experimental and clinical applications of flow cytometric analysis in medical practice are in the related fields of clinical immunology and hematology for a variety of tasks involving blood cell counting and classification. Applications are also being pursued in the fields of genetics, microbiology, oncology, parasitology, pharmacology, and toxicology.

Various types of commercial flow cytometers are available, but most of their basic functions are similar and can be described as follows. Single cells in liquid suspension are carried through a sensing device commonly illuminated by laser beams. In immunofluorescence FCM, fluorochrome molecules attached to antibody molecules bound to the biological particles are excited at a particular wavelength of light as the particles pass through a laser beam. The fluorochromes subsequently decay and emit light of another wavelength which is detected by the optical device. Light scattering, collected over one or more angular ranges, is determined and the light is converted into electronic signals by photomultiplier tubes (PMTs). Electronic signals whose magnitudes are proportional to the intensity of the emitted and scattered light are amplified and evaluated.

Fluorescein isothiocyanate (FITC) is generally used as a fluorochrome, and many antibodies conjugated to FITC are available commercially. Phycoerythrin (PE), another fluorochrome in general use, is excited at the same wavelength as FITC (Table I), thus providing a method of two-color FCM and FACS. PE labeling is more technically complex than FITC labeling and may not be practical for many laboratories. However, many

TABLE I Fluorochromes Used in FCM

Fluorochrome	Peak wavelength (nm)		Light source
	Absorption	Emission	
Fluorescein	495	530	Argon laser (488)
Rhodamine	555	620	Mercury arc (546)
B-Phycoerythrin	546	575	Mercury (546)
R-Phycoerythrin	490, 546	575	Argon (488)
Texas Red	568	590	Krypton laser (576)
Allophycocyanin	650	660	Helium-Neon laser (633)
Propidium iodide	490	585	Argon (488)

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or in cases of two-, thre

II. INSTRUMENTATI

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model of laser is usuall
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offered are argon, krypt
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are less expensive to pu
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secondary antibodies labeled with PE are commercially available. Texas Red and allophycocyanin require a second laser since their excitation wavelengths differ from those of FITC and PE. They are generally used when autofluorescence is a problem (e.g., with long-term cell cultures) or in cases of two-, three-, or four-color analyses.

II. INSTRUMENTATION

An optical flow cytometer includes a light source, light collection optics, and illumination optics similar to the objectives of a light microscope. Flow cytometers, however, require more intense light sources than commonly used in light microscopes since a cell passing through the instrument is typically illuminated for less than 10 μsec. This requirement for an intense light source is met by arc lamps (high-pressure mercury and xenon lamps) or lasers.

A variety of instruments is commercially available worldwide (Table II). For analytical cytology, two major classes of instruments are available: flow cytometers and cell sorters. In general, a flow cytometer measures the physical and chemical characteristics of cells in a flowing liquid suspension, whereas a cell sorter can also selectively separate different cell populations. The two most important components of a flow cytometer are the laser or arc lamp and the flow chambers. The type and model of laser is usually specified by the manufacturer since lasers from different manufacturers have different sizes and shapes. Those currently offered are argon, krypton, xenon, and helium–neon ion lasers and dye lasers. In many flow cytometers, the excitation source consists of a high-pressure mercury or xenon arc lamp. Compared with lasers, arc lamps are less expensive to purchase and maintain, and are much smaller and easier to incorporate into instruments. However, they possess several disadvantages, such as lower light output at all wavelengths, less selectivity of excitation wavelength, relatively short life-span of the lamp, more difficulty in shaping and transporting the beam, and no possibility for measuring light scatter at small angles. The different commercial instruments also have different fluidics and optical arrangements. Buyers are urged to contact various manufacturers for detailed information and compare products before purchase.

Although commercial flow cytometers are equipped with various computer systems that collect, store, and analyze FCM data, the data received by these systems are essentially the same. The level of fluorescence obtained from the electronic sensors corresponds to the amount of scattered or fluorescent light and is translated into digitized readings either in linear or logarithmic fashion. These output voltages are recorded in 0.01-V increments (channels) from which histograms are constructed. It

Light source
Argon laser (488)
Mercury arc (546)
Mercury (546)
Argon (488)
Krypton laser (576)
Helium-Neon laser (633)
Argon (488)

TABLE II Commercial Flow Cytometers

Source	Comments
Becton Dickinson Immunocytochemistry Systems 2375 Garcia Avenue P.O. Box 7375 Mountain View, CA 94039 800-821-9796	Becton Dickinson Immunocytometry Systems include the FACS Analyzer and FACScan and the latest versions of the Fluorescence Activated Cell Sorter (FACS®) which are called the FACStar^{PLUS}. The Becton Dickinson cell sorters use a water-cooled argon ion laser source (2–5 Watts), whereas the FACS Analyzer and FACScan are nonsorting flow cytometers that use a mercury arc lamp and a Cyonics air-cooled argon ion laser.
Coulter Corporation Epics Division P.O. Box 4486 Hialeah, FL 33014-0486 305-885-0131	EPICS® instruments are laser source flow cytometers with an orthogonal geometry. The EPICS V is designed primarily for research laboratory use, whereas EPICS C and EPICS Profile are intended for clinical applications. The standard laser in the EPICS research instruments is a water-cooled 4-W argon laser with enhanced UV output. A second ion laser and/or dye laser can be added for dual-beam illumination. The EPICS C is a single beam sorting instrument using a 2-W argon laser. The EPICS Profile is a nonsorting bench-top flow cytometer system designed for routine clinical laboratory applications and the standard light source is an air-cooled Omnichrome argon laser emitting about 25 mW at 488 nm.
Kratel, SA 64 Ch. de St. Maurice/BP82 CH-1222 Geneve-Vesenaz Switzerland (022) 52 33 74	The Kratel Partograph flow cytometers include the Partograph FMP Analyzer and Partograph FMP Sorter. The Partograph FMP is available with low power (15 mW) argon and/or He–Ne laser sources. An arc lamp source and sorting capability are also offered.
ODAM 34 Rue de L'Industrie 67160 Wissenbourg France 94 99 32	The ODAM ATC 3000 flow cytometer is a multiparameter cell sorter equipped with argon and krypton ion lasers or dye lasers in a dual system.
Skatron A/S P.O. Box 8 N-3401 Lier Norway (03)850770	The Argus flow cytometer is the recent commercial version of the arc source instrument: Argus Flow Microphotometer is equipped with a 100-W mercury arc lamp. An argon laser is optional.

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ments are made on each
set of measurements can
Data are normally coll
(cell internal complexity)
Data analysis can use ma
including single paramet
as a function of the signal
and dot displays showir
measurements by two F
centage of cells falling w
the means of signal leve
One-parameter FCM
number of cells with a
signal strength. The y ax
channel whereas the x a
resulting bar graph is u
connected to form a bro
the line reflects the total
possible to visually estir
a mixed population.
Typical two-paramete
frequency of cells for tw
each parameter are prese
number of dots in each
plots are similar to a geol
lines are used to define
Both linear and logarit
flow cytometers. The ch
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cell populations.

III. APPLICATIONS

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is desirable in some systems, especially when more than two measurements are made on each cell, to collect data in list mode so that the full set of measurements can be repeatedly processed for optimal results.

Data are normally collected for forward scatter (cell size), side scatter (cell internal complexity), and one or more fluorescence wavelengths. Data analysis can use many forms of display and numerical evaluation, including single parameter histograms in which cell counts are plotted as a function of the signals collected by a single sensor and contour plots and dot displays showing cell frequency distribution as a function of measurements by two FACS sensors. Sample statistics, such as the percentage of cells falling within specified ranges of sensor readings and the means of signal levels, can also be acquired.

One-parameter FCM data can be displayed simply by plotting the number of cells with a particular signal strength as a function of the signal strength. The y axis shows the number of cells falling into each channel whereas the x axis is usually divided into 256 channels. The resulting bar graph is usually drawn with only the tops of the bars connected to form a broken line, where the height under any part of the line reflects the total count of cells in a particular channel. It is thus possible to visually estimate the relative frequencies of each cell type in a mixed population.

Typical two-parameter FCM displays contain information about the frequency of cells for two measurement parameters. Channel values for each parameter are presented on the x and y axes. In a dot display, the number of dots in each region simply indicates the cell count. Contour plots are similar to a geological survey topographic map in which contour lines are used to define specified cell frequency levels.

Both linear and logarithmic signal amplifications are available for most flow cytometers. The choice of which to use has important implications for the display and analysis of FACS data. Although both logarithmic and linear data displays contain the same information, linear amplification is appropriate for signals with a limited dynamic range and for signal distributions that include narrow peak components, such as DNA data. In contrast, logarithmically processed data can cover a wide dynamic range at constant relative resolution and are suitable for some biological measurements such as the distribution of a cell-surface antigen among cell populations.

III. APPLICATIONS

The power of flow cytometric analysis lies in its ability to make quantitative multiparameter measurements on a large number of individual cells. Any of a number of cellular properties can be detected with a fluorescent

52 33 74

ODAM
34 Rue de L'Industrie
67160 Wissenbourg
France
94 99 32

The ODAM ATC 3000 flow cytometer is a multiparameter cell sorter equipped with argon and krypton ion lasers or dye lasers in a dual system.

Skatron A/S
P.O. Box 8
N-3401 Lier
Norway
(03)850770

The Argus flow cytometer is the recent commercial version of the arc source instrument. Argus Flow Microphotometer is equipped with a 100-W mercury arc lamp. An argon laser is optional.

label or antibody; that measurement, in combination with other cytological properties, provides a sophisticated characterization of the cell. Particularly with the advent of monoclonal antibody technology, FCM has provided a method of rapid quantitative analysis of multiple cellular antigens.

Examples of FCM applications of importance in cell biology include quantitative cell-cycle analysis, flow karyotyping and chromosome sorting, flow cytometry and sorting of sperm cells, virus–cell interaction analysis, and measurements of intracellular ionized calcium concentration and membrane potential. In basic and clinical immunology, FCM has been important for correlation of changes in particular cell subpopulations with the onset of clinical disease, investigation of T and B lymphocyte developmental pathways, identification of functional characteristics of lymphocyte subpopulations, and, most recently, studying the regulation of gene expression. In the field of hematology, FCM has contributed to analysis and sorting of normal and malignant blood and bone marrow cells.

IV. PROCEDURES

For further reference, the reader should consult Shapiro (1988); Parks *et al.* (1989); Melamed *et al.* (1990); Coligan *et al.* (1991); and Robinson (1993).

A. Preparation of Reagents

Lysis Reagent

NH_4Cl	8.2 g
$NaHCO_3$	0.84 g
EDTA	0.37 g
Sterile distilled water	1 liter

Adjust pH to 7.2; store at 4°C for 1 mo.

2% Paraformaldehyde

Paraformaldehyde	10 g
Balanced salt solution	500 ml

Warm to 60°C to improve solubility. Adjust pH to 7.2 with NaOH before adjusting final volume to 500 ml. Store in the dark at 4°C for 1 mo.

10% Sodium Azide

Sodium azide
Balanced salt solutio

Store at room tempe

FCM Buffer

10% Sodium azide
Heat-inactivated feta
Balanced salt solutio

Store at 4°C for 1 m
substituted for fetal

Sheath Fluid

Normal saline (0.85
filtered.

B. Preparation of Single

1. Preparation of Cells from

1. Following the protocol wet with ethanol to s lymph nodes.
2. Place organ(s) in a ste Hank's balanced salt s
3. Disrupt the tissue by syringe plunger or aspi
4. Transfer to a 100-μm the resulting single ce volume to 10 ml with
5. Centrifuge at 2000 rpn
6. Wash the pellet twice centrifuging as in Step
7. Resuspend cells in cult

2. Preparation of Tumor Ce

1. Cell dissociation for flo for preservation of cell depend on the nature

nation with other cytologi-
terization of the cell. Partic-
ody technology, FCM has
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ing and chromosome sort-
ells, virus–cell interaction
onized calcium concentra-
linical immunology, FCM
in particular cell subpopu-
igation of T and B lympho-
f functional characteristics
ently, studying the regula-
logy, FCM has contributed
nt blood and bone marrow

ılt Shapiro (1988); Parks *et*
al. (1991); and Robinson

ust pH to 7.2 with NaOH
. Store in the dark at 4°C

10% Sodium Azide

Sodium azide	10 g
Balanced salt solution	100 ml

Store at room temperature for 2 mo.

FCM Buffer

10% Sodium azide	10 ml
Heat-inactivated fetal calf serum	30 ml
Balanced salt solution	1 liter

Store at 4°C for 1 mo. Bovine serum albumin (1%, w/v) may be substituted for fetal calf serum.

Sheath Fluid

Normal saline (0.85%) made in double-distilled water and triple filtered.

B. Preparation of Single Cell Suspensions

1. *Preparation of Cells from Lymphoid Organs of Animals*

1. Following the protocol established for humane sacrifice of the animal, wet with ethanol to sterilize and remove spleen, thymus, and/or lymph nodes.
2. Place organ(s) in a sterile petri plate containing a small volume of Hank's balanced salt solution (HBSS) with 5% fetal calf serum.
3. Disrupt the tissue by mincing with scissors, then mashing with a syringe plunger or aspirating up and down with a syringe and needle.
4. Transfer to a 100-μm screen and filter to remove clumps. Transfer the resulting single cell suspension to a centrifuge tube and bring volume to 10 ml with HBSS.
5. Centrifuge at 2000 rpm for 5 min.
6. Wash the pellet twice by resuspending the cells in 10 ml HBSS and centrifuging as in Step 5.
7. Resuspend cells in culture medium and count by trypan blue exclusion.

2. *Preparation of Tumor Cells*

1. Cell dissociation for flow cytometry should be achieved enzymatically for preservation of cell morphology and function. Enzymes used will depend on the nature of the tumor, and some experimentation may

be needed. Suggested enzymes include trypsin, collagenase, neutral protease, DNase, and hyaluronidase, or combinations thereof. In general, sarcomas are more easily digested than carcinomas.

2. Follow the procedure given for single cell suspensions. After the tissue is minced with scissors, incubate with appropriate enzyme(s) for 1 hr. Filter out clumps and continue as in Procedure 1.

3. *Preparation of Leukocytes by Lysis of Erythrocytes*

1. Obtain blood sample, or prepare a single cell suspension of lymphoid organ cells.
2. Pellet cells by centrifugation and resuspend in 5 ml lysing reagent per organ, per 2 ml blood, or per 5–10 × 10^7 viable lymphocytes.
3. Incubate 10 min at room temperature with gentle mixing.
4. Fill tube with HBSS and centrifuge at 2000 rpm for 5 min. Wash the pellet with HBSS twice and resuspend cells in culture medium.

4. *Ficoll–Hypaque Isolation of Mononuclear Cells*

1. If using whole blood, dilute in an equal volume of HBSS, or prepare a single cell suspension of organ cells in HBSS.
2. Place 4 ml Ficoll–Hypaque in 17 × 100-mm tubes.
3. Overlay carefully with cell suspension, 5–6 ml per tube.
4. Centrifuge at 1800 rpm at room temperature for 20 min.
5. Transfer lymphocytes from the interface to an appropriately sized tube and dilute at least 50% in HBSS.
6. Centrifuge at 2000 rpm for 5 min. Wash cells twice with HBSS by resuspending pellet in 10 ml or more of HBSS and centrifuging at 2000 rpm for 5 min.

C. Basic Cell Staining Procedure

1. *Antibodies*

1. For detailed protocols to conjugate antibody to FITC or biotin, see Chapter 9. To conjugate antibody to Texas Red, follow the FITC labeling procedure using 1 mg/ml Texas Red in cold anhydrous acetonitrile.

2. Use fluorochrome-conj
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taining a spacer betwee
to prevent precipitatior
3. For biotin-conjugated a
bovine serum albumin

2. *Cell Staining*

1. Keep all cells, antibod
capping of antibodies.
2. Prepare a single cell su
in cell culture mediun
3. Determine viable cell
4. Centrifuge cell suspen
supernatant. Resuspe
on ice.
5. Distribute 50 μl cell s
a microtiter plate.
6. Add 50 μl antibody t
antibody will be FITC
antibody is unlabeled.
predetermined by test
A few unstained contr
cytometer settings.
7. Mix antibody with c
30 min, depending or
8. Centrifuge plates or t
flick once to discard s
9. Wash the cells with 1
gently mixing the buff
as in Step 8.
10. If appropriate, add 5
(with specificity for th
samples from Step 6 t
body to cells. Incubat
11. Resuspend in 500 μl
microtiter plates, resu

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d in 5 ml lysing reagent
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ume of HBSS, or prepare
BSS.
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IBSS and centrifuging at

dy to FITC or biotin, see
as Red, follow the FITC
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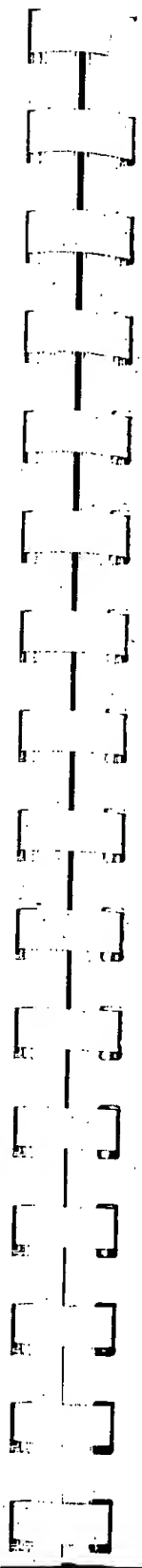


2. Use fluorochrome-conjugated avidin to detect biotin-labeled antibody. For some applications, it may be necessary to use biotin containing a spacer between the protein-binding and avidin-binding sites to prevent precipitation.
3. For biotin-conjugated antibodies, use FCM buffer prepared with 1% bovine serum albumin instead of fetal calf serum.

2. *Cell Staining*

1. Keep all cells, antibodies, and buffers on ice at all times to prevent capping of antibodies.
2. Prepare a single cell suspension of cells as described earlier and place in cell culture medium on ice.
3. Determine viable cell count by trypan blue exclusion.
4. Centrifuge cell suspension for 8 min at 1800 rpm and 4°C, and discard supernatant. Resuspend in FCM buffer at 2×10^7 cells/ml and keep on ice.
5. Distribute 50 μl cell suspension to small tubes (13-mm) or wells of a microtiter plate.
6. Add 50 μl antibody to each tube or well. For a direct assay, this antibody will be FITC labeled; for an indirect assay, the primary antibody is unlabeled. Appropriate dilutions of antibody should be predetermined by testing intensity of staining with several dilutions. A few unstained controls should be prepared for optimizing the flow cytometer settings.
7. Mix antibody with cells by shaking gently. Incubate on ice 20–30 min, depending on the affinity of the antibody.
8. Centrifuge plates or tubes 5 min at 1800 rpm and 4°C. Decant or flick once to discard supernatant.
9. Wash the cells with 100–200 μl ice-cold FCM buffer 2–3 times by gently mixing the buffer with cells, followed by 5 min centrifugation as in Step 8.
10. If appropriate, add 50 μl FITC-labeled avidin or second antibody (with specificity for the primary antibody). Include half of the control samples from Step 6 to measure nonspecific binding of second antibody to cells. Incubate 20–30 min on ice. Wash as in Step 9.
11. Resuspend in 500 μl ice-cold FCM buffer. If staining was done in microtiter plates, resuspend in 100 μl FCM buffer and then add to

tubes containing 400 μl ice-cold FCM buffer. Keep on ice until flow cytometry.

12. If cells will be stored before running, fix by resuspending in 100 μl FCM buffer and, while vortexing, adding 100 μl 2% paraformaldehyde solution. Keep samples at 4°C for up to several days.

D. Two-Color Cell Staining

The steps for two-color staining are the same as those for single color staining, with the following exigencies.

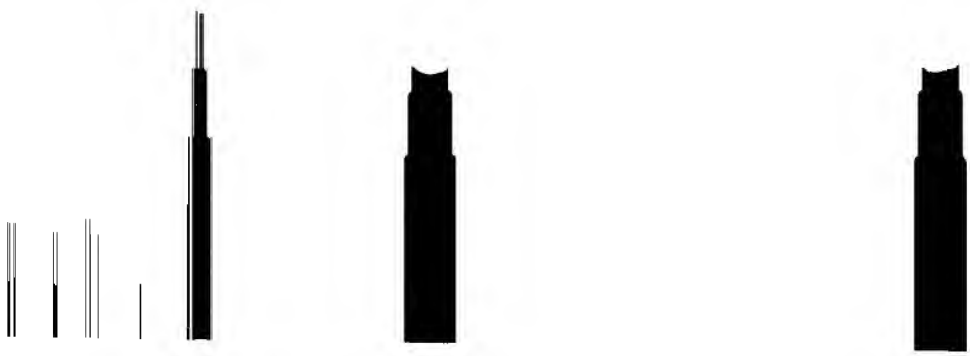
1. When direct staining, both antibodies may be added simultaneously if there will be no interactions between them.
2. When using one direct and one indirect antibody, add the indirect primary antibody first. If both antibodies are of the same species, it may be necessary to perform a blocking step before adding the direct antibody. To do this, after the excess secondary antibody has been washed off, an excess of purified immunoglobulin of the same species as the primary antibody is added and incubated for 10 min prior to adding the antibody specific for the surface antigen. The excess immunoglobulin occupies any unused binding sites of the secondary antibody.
3. When both antibodies will be indirectly labeled, the assay must be arranged to avoid any possibility of interactions between them.
   a. If the primary antibodies are of different species, they may both be added during the first incubation. Then the secondary antibodies specific for each species may be added during the second incubation, as long as they will not bind each other or cross-react with other species.
   b. If any of the antibodies are of the same species, it will be necessary to conduct sequential incubations. Adjust the order in which antibodies are added to avoid interactions and use blocking steps (Step 2) as necessary.
4. Prepare control samples that are singly stained with each fluorochrome to use when adjusting the flow cytometer. If a dual-positive control is available, it should be stained with both fluorochromes.

E. Operation of Flow Cytometer

The instructions here are generalized and must be used in conjunction with the manufacturer's instructions.

1. Turn on machine 20 r
2. Check levels of sheath as necessary. Add a s for disinfection.
3. Flush air from the tub
4. Run the wash cycle u: by distilled water or sh be included in these w than 200 events per m
5. When laser is warmec ment. Some alignmen texed prior to running er's directions. On the alignment beads shou fluorescence peaks sh the signal while minir for the batch of fluores dinates may be saved for subsequent runs.
6. Run a sample of unst cells/sec or within ma
7. Adjust the forward sca The choice between li all parameters may be cell population. Linea neous cell populations range is limited. Mor neous cell population compress the range of
8. Adjust side scatter PM sary to achieve good s ple, for example, to d lymphocytes.
9. Adjust PMT voltages stained cells, adjust PM that the peak of unst histogram or on the fa amplification gains, th rithmic gains of FL1 a and compress the scal

1. Turn on machine 20 min to 1 hr before use to warm up the laser.
2. Check levels of sheath fluid and waste in reservoirs; empty and fill as necessary. Add a small amount of bleach to the waste reservoir for disinfection.
3. Flush air from the tubing.
4. Run the wash cycle using 5% bleach to degrade proteins, followed by distilled water or sheath fluid. Manufacturer's cleansers may also be included in these washes. Following wash, there should be fewer than 200 events per min.
5. When laser is warmed up, run fluorescent beads and check alignment. Some alignment beads specify that they should not be vortexed prior to running. Adjust alignment according to manufacturer's directions. On the forward scatter vs side scatter histogram, the alignment beads should fall in a tight cluster. The variability of the fluorescence peaks should be small (1–2%). The aim is to maximize the signal while minimizing variability. Once the ideal parameters for the batch of fluorescent beads have been established, these coordinates may be saved and used to adjust the alignment of the beads for subsequent runs.
6. Run a sample of unstained cells and adjust flow rate to 1000–2000 cells/sec or within manufacturer's recommended parameters.
7. Adjust the forward scatter amplification gains to exclude cell debris. The choice between linear and logarithmic amplification gains for all parameters may be made on the basis of the homogeneity of the cell population. Linear amplifications are appropriate for homogeneous cell populations or for cell cycle analyses in which the intensity range is limited. More information may be obtained on heterogeneous cell populations by using logarithmic amplification gains to compress the range of intensity.
8. Adjust side scatter PMT voltages and amplification gains as necessary to achieve good separation of different cell types within a sample, for example, to distinguish granulocytes from monocytes and lymphocytes.
9. Adjust PMT voltages and amplification gains. While running unstained cells, adjust PMT voltage of fluorescence 1 (FL1) and FL2 so that the peak of unstained cells falls either off the left side of the histogram or on the far left side, as preferred. If using logarithmic amplification gains, these parameters may not be adjustable. Logarithmic gains of FL1 and FL2 will increase the scale of weak signals and compress the scale of strong signals.



10. While running stained cells, adjust PMT voltages of FL1 and FL2 to achieve good separation from the peak of unstained cells.
11. Run stained cell sample and adjust regions, bitmaps, or gates. Bitmaps can be drawn around each type of cell in a nonhomogeneous cell population, such as whole blood. Alternatively, if only one cell population is used, bitmaps may be drawn in concentric rings to compare those cells that are most alike as well as the larger population containing more variability. Likewise, regions of interest in single-parameter histograms may be adjusted to include various proportions of the peak from all the cells to only those that stain most intensely.
12. Run the test samples.
13. Run the wash cycle as in Step 5.
14. Initiate the shutdown process for the machine.
15. Gates, bitmaps, and regions are used to identify cells of interest. In general, gates refer to one-dimensional thresholds whereas bitmaps are two-dimensional. These parameters are applied to data acquisition; regions are thresholds applied to data analysis. Gates on data acquisition mean that only cells of interest will be included in the cell count. For example, it is desirable to set a gate on forward scatter or side scatter (or both) to exclude cell debris. Gates and bitmaps can also be set to include only the cells of interest from a mixed cell population. It is often helpful to set a cut-off for fluorescence obtained by examining unstained cells, or to determine the fluorescence produced by nonspecific binding of an irrelevant antibody or the secondary antibody of an indirect procedure.

F. Propidium Iodide Elimination of Dead Cells

1. Make 25–50 μg/ml propidium iodide (PI) in PBS.
2. Stain cells and optimize machine for single staining protocol, as described.
3. Run unstained sample, and set up FL1 (FITC) vs FL2 (PI) histogram.
4. Adjust PMT of FL2 so cells appear in lower left quadrant (perhaps 10–20 V less than FL1).
5. Run FITC-stained sample. Adjust compensation by increasing the setting for %FL1 in FL2. This increases the %FL1 that is subtracted from the FL2 sensor, until the histogram shows no higher readings for FL2 in stained than unstained cells.

6. Add 10 μl PI soluti
control sample.
7. Set a gate for FL2 to
the dead PI-stained c
8. Add 10-μl PI to FITC
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9. Add 10 μl PI to each
10. After running all san
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G. Two-Color FCM Ana

1. Stain cells with FITC
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2. Warm up machine a
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3. Run unstained samp
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5. Run FITC-stained co
%FL1 in FL2 setting
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6. Run the PE-stained
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7. Run the dual-positiv
PE. Confirm that the
vs FL2 histogram.
8. Mix the single-staine
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9. Run samples.

V. PITFALLS AND CO

Optimal labeling of antib
agents used. All reagents
Texas Red labeling is mc

6. Add 10 μl PI solution to unstained control. Vortex and run the control sample.
7. Set a gate for FL2 to include the unstained control cells but exclude the dead PI-stained cells.
8. Add 10-μl PI to FITC-stained sample. Vortex and run. Confirm the validity of the FL2 gate for the positive cells.
9. Add 10 μl PI to each sample immediately prior to running it.
10. After running all samples, be sure to run the wash cycle using 5% bleach, followed by distilled water, to clean excessive dye from the tubing.

G. Two-Color FCM Analysis

1. Stain cells with FITC and PE, following the two-color staining protocol. In addition, make single-stained control samples for both FITC and PE, using cells and antibodies that stain most brightly. If possible, stain a dual-positive control sample with FITC and PE.
2. Warm up machine and adjust for fluorescent beads, controls and unstained cell parameters, and PMT voltages for FL1 and FL2.
3. Run unstained sample, and set up FL1 vs FL2 histogram.
4. Adjust FL2 so cells appear in lower left quadrant.
5. Run FITC-stained control. Adjust compensation by increasing the %FL1 in FL2 setting until the FL2 reading is no more than for unstained cells.
6. Run the PE-stained control. Adjust compensation of %FL2 in FL1 until the FL1 is no more than for unstained cells.
7. Run the dual-positive control sample stained with both FITC and PE. Confirm that the cells fall in the upper right quadrant of the FL1 vs FL2 histogram.
8. Mix the single-stained control samples from Steps 5 and 6 and run to insure that no dual-positives are detected.
9. Run samples.

V. PITFALLS AND CONCERNS

Optimal labeling of antibodies with fluorochromes depends on the reagents used. All reagents of FITC and Texas Red should be made fresh. Texas Red labeling is more difficult than labeling with FITC or biotin,

since it is more dependent on the isotypes and species of antibody to be labeled. The ability of specific antibodies to withstand the conjugation procedures and retain activity should also be considered.

Obtaining high specific signal with minimal background fluorescence requires reducing background fluorescence caused by nonspecific binding and autofluorescence. Autofluorescence often occurs with cultured cells and can be reduced by using cells in an exponential growth phase, appropriate optical filters, or reagents that have been conjugated with red fluorochromes. Unstained cells should be included as controls to assess background autofluorescence. A high fluorochrome-to-protein ratio in the conjugation procedure can improve signal-to-noise ratios. Fixation of cells in dilute solutions of paraformaldehyde may increase the nonspecific background fluorescence.

All experiments should include negative controls labeled with irrelevant antibodies to determine nonspecific binding of any labeled reagent to the cell population under examination. For indirect staining, either omit the first antibody or use an irrelevant antibody of the same isotype to establish background fluorescence or nonspecific binding.

The intensity and specificity of staining can be influenced by several factors. It is important to include sodium azide in the staining buffer, and all the reagents and staining tubes should be kept on ice to decrease metabolic activity and inhibit the capping phenomenon. The optimum predetermined antibody concentration should be used in all staining procedures. All antibody preparations should be free of aggregates to avoid nonspecific binding to the Fc receptor present on the surfaces of some cells. Most secondary antibodies are polyclonal antisera that have been affinity purified. Optimum concentrations for each secondary reagent must be determined for each primary antibody used in each assay.

The choice of direct or indirect staining procedures requires a tradeoff. Direct staining of cell-surface markers with specific antibodies conjugated to FITC provides a more specific but less intense staining reaction. Indirect procedures, involving primary antibodies against cell-surface markers and secondary antibodies conjugated to FITC and directed against the primary antibody (usually in a species-specific manner), provide more intensity but with a loss of specificity. Signal intensity may also be enhanced by conjugating the antibody to biotin and using FITC-conjugated avidin.

Cross-reactions are most problematic in multicolor staining systems in which two primary antibodies produced in different species are detected with species-specific secondary antibodies labeled with different fluorochromes. Controls in which each second-step reagent is tested on both primary antibodies individually must be included. The order of

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adding reagents is critical in multicolor staining. In a protocol in which both direct and indirect staining are performed, the indirect is done first. Every multicolor staining procedure should include single color controls in which cells are stained with each fluorochrome-labeled reagent separately, then compared with a sample stained with multicolor-labeled reagents. Single-color controls are used for setting compensation in flow cytometric analysis.

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